PCT/EP 98/06183

EP 98/06183

09/509391

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

98201411.0

PRIORITY DOCUMENT

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 98201411.0
Demande n°:

Anmeldetag:
Date of filing: 01/05/98
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
Academisch Ziekenhuis bij de Universiteit van Amsterdam
1105 AZ Amsterdam ZO
NETHERLANDS

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Recombinantly prepared antimicrobal proteins TC-1 and TC-2 or variations thereof

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
/

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

RECOMBINANTLY PREPARED ANTIMICROBIAL PROTEINS TC-1 AND TC-2 OR VARIATIONS THEREOF.

1. Characterization of TC-1 and TC-2.

Bactericidal proteins thrombocidin-1 (TC-1) and –2 (TC-2), purified from human blood platelets, were analyzed by MALDI and electrospray (ES) mass spectrometry.

ES analysis of TC-1 (fig 1a) yielded a molecular weight of 7436,3 ±1,3 Da. Analysis by MALDI (fig 2) also revealed a peak of this size, next to an additional number of peaks with M+1 of 7107.2, 7227.7 and 7602.0 Da. The molecular weights of these proteins can be explained by assuming that these proteins are C-terminal truncation products of NAP-2 (fig 4): the calculated molecular weights correspond well with the values experimentally determined (Table 1). These data suggest that TC-1 is a mixture of C-terminally truncated forms of NAP-2. The 7436 Da protein seems to be the main component. We designated this protein TC-1*.

ES spectroscopy of TC-2 (fig 1b) yielded a molecular weight of 9100,5 ± 1,3. This value corresponds to the calculated molecular weight (Table 1) and was confirmed by MALDI-tof spectroscopy. In addition to TC-2, only one minor contamination was present (10081 Da, fig 3).

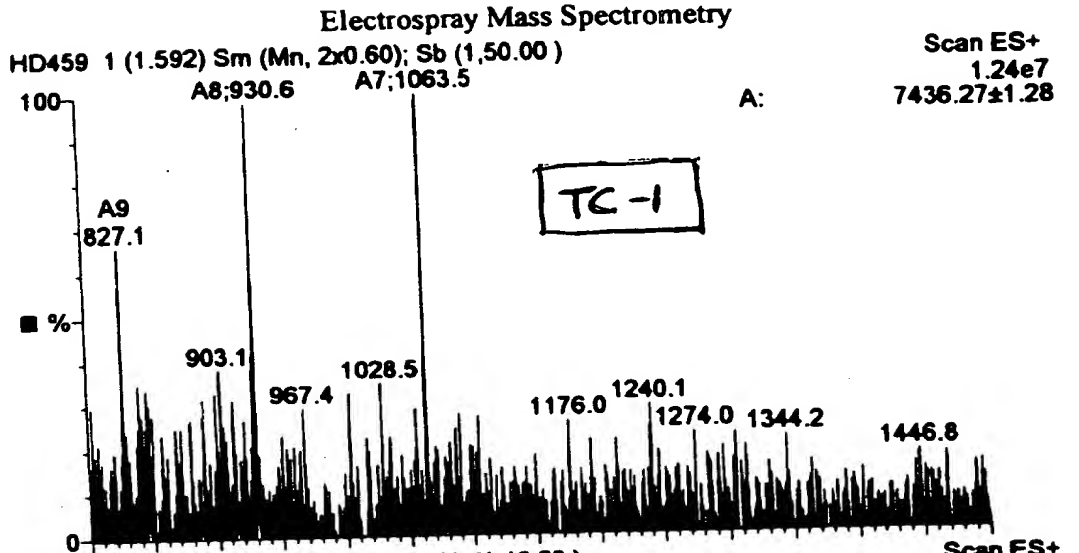
Partial sequencing of TC-2 indicated that the N-terminus of TC-2 is identical to that of CTAP-III. Based on the mass-spectrometrical data (figs 1b and 3) however, it appears that the mass found experimentally was smaller than the mass of CTAP-III (Table 1). This can be explained by assuming that TC-2 is truncated C-terminally and misses 2 amino acids compared to CTAP-III.

Thrombocidins identified thus far are indicated in fig 4, together with the sequences of CTAP-III and NAP-2.

H 01.05.98

Electrospray Mass Spectrometry

1 A



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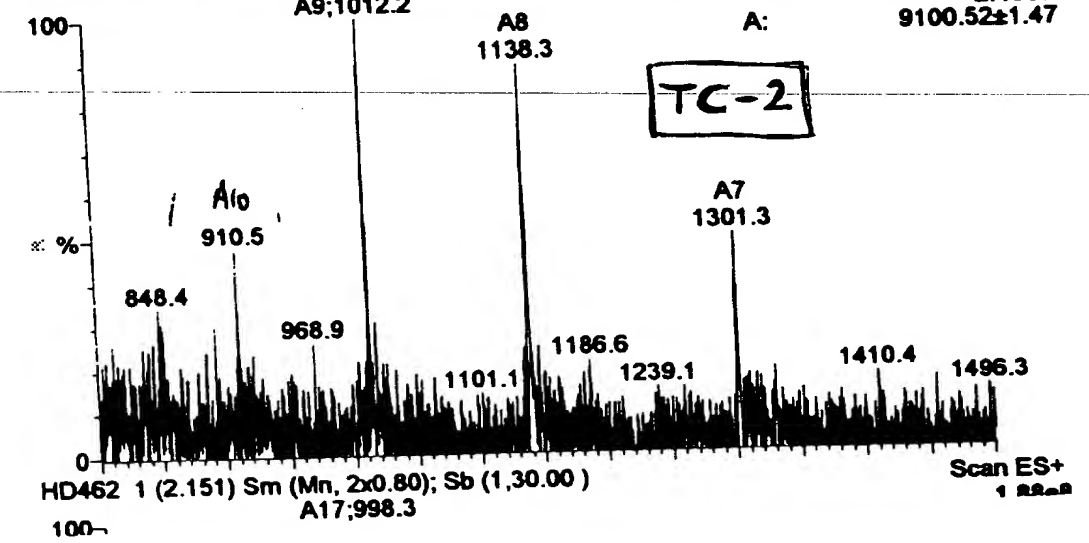

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01-05-1998

Savitzky-Golay Order = 2 Points = 55

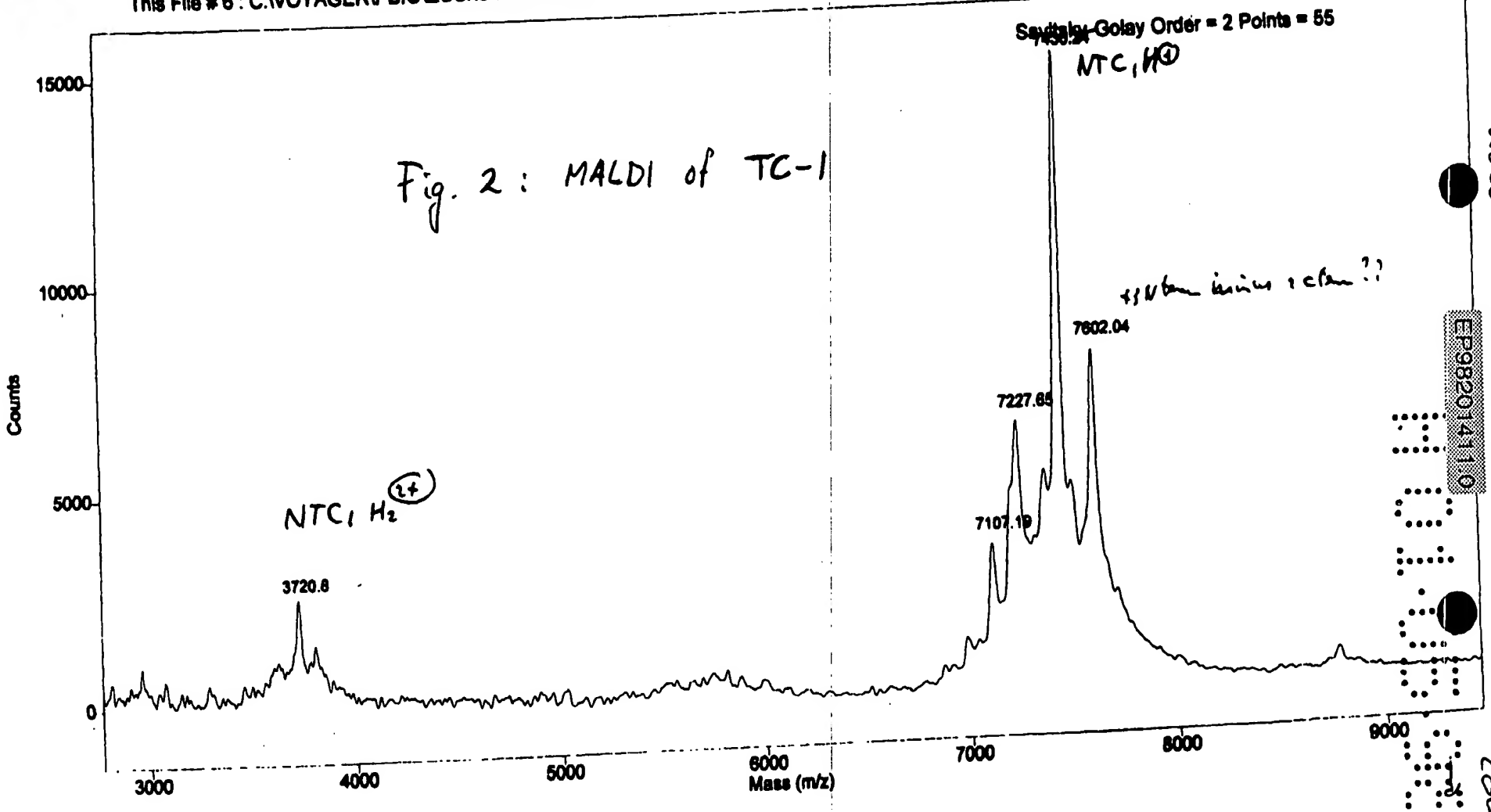

Fig. 2 : MALDI of TC-1

EP98201411.0

DESC

Comment: NTC1/a-cyano 1+1

Method: LDE1002

Mode: Linear

Accelerating Voltage: 25000

Grid Voltage: 88.000 %

Guide Wire Voltage: 0.150 %

Negative Ions: OFF

Laser : 1900

Scans Averaged: 33

Pressure: 9.50e-07

Low Mass Gate: 1000.0

Mirror Ratio: 1.060

PSD Mirror Ratio:

Timed Ion Selector: 15.7

Delay Time:150ns

bovins . usq .

MALDI-TOF

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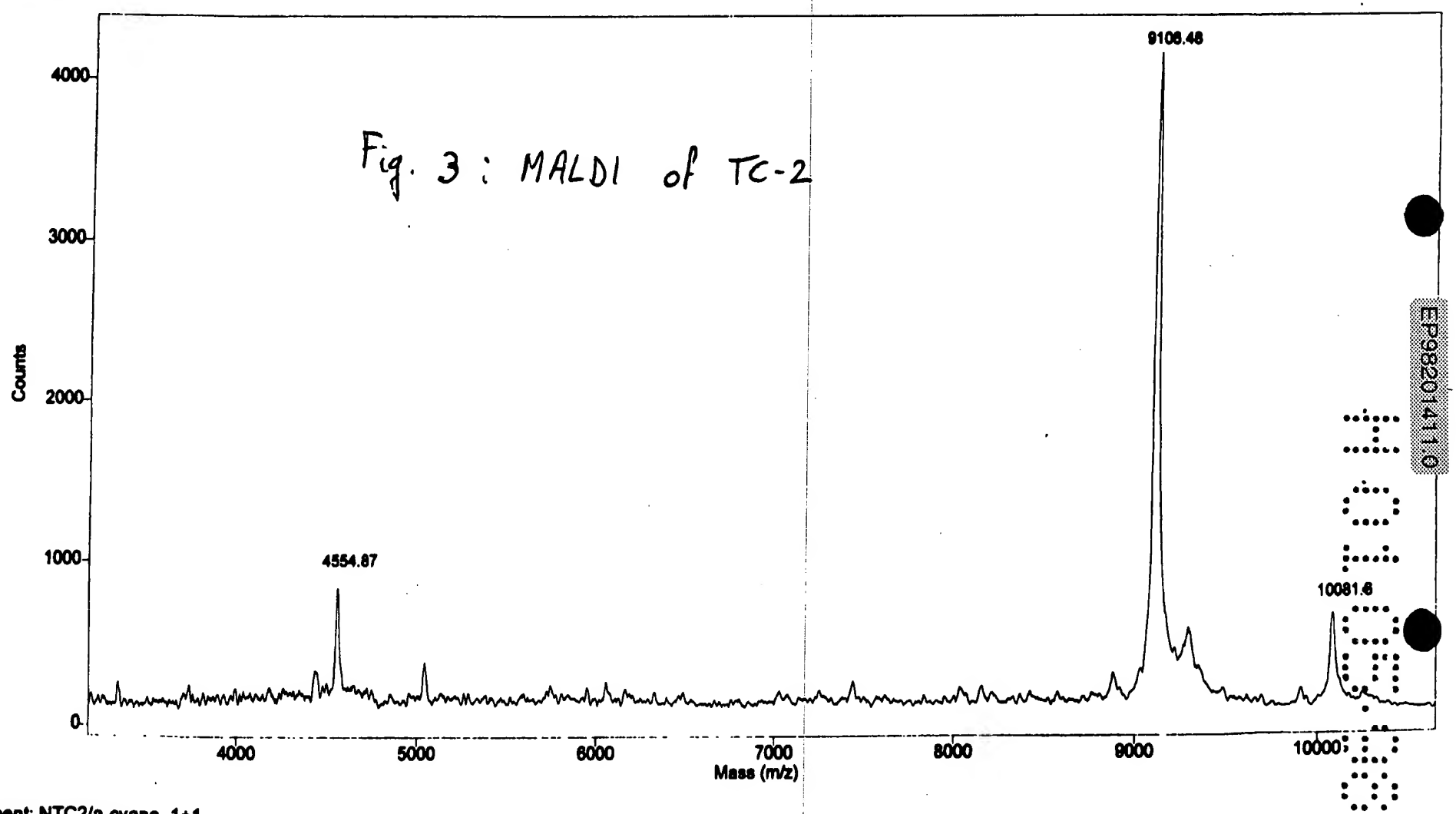

Fig. 3 : MALDI of TC-2

Comment: NTC2/a-cyano 1+1

Method: LDE1002

Mode: Linear

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Guide Wire Voltage: 0.150 %

Negative Ions: OFF

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Scans Averaged: 101

Pressure: 8.54e-07

Low Mass Gate: 1000.0

Mirror Ratio: 1.060

PSD Mirror Rati :

Timed Ion Selector: 15.7

Delay Time:150ns

CTAP-III:	NLAKG KEESL DSDLY AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD
NAP-2:	AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD
TC-1:	LRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD
TC-1*:	AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DES
TC-2:	NLAKG KEESL DSDLY AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DES

Fig 4. Sequences of thrombocidins and related proteins.

Table I. Interpretation of mass-spectrometrical data of TC-1 and TC-2: comparison with CTAP-III.

Component	Mol. weight (Da) MALDI/ES	Calc.	Sequence of N-terminus	C-terminus
CTAP-III		9287,2	NLAKGKEESDSDLYAELR	....AGDESAD
TC-1a	7106,2	7105,8	AELR	....AG
TC-1b	7226,7	7220,9	AELR	....AGD
TC-1*	7436,3	7437,5	AELR	....AGDES
TC-1d	7601,0	7600,7	YAELR	....AGDES
TC-2	9100,5	9101,6	NLAKGKEESDSDLYAELR	....AGDES

*: main component

2. Antibacterial activity of thrombocidins and thrombocidin-derived peptides.

2.1 Methods

The experimental set-up for testing antibacterial activity of thrombocidins was as follows.

Bacteria from blood agar plates were grown overnight in tryptic soy broth (TSB), subcultured in fresh TSB and grown to log-phase in 2-3 hours. Bacteria were pelleted, washed once in 10mM phosphate buffer (pH 7,0) + 1% TSB (v/v) and resuspended in the same medium to an OD620 of 0,1. This suspension was further diluted 200 (*B. subtilis*) or 500 times (*E. coli* and *S. aureus*) to obtain suspensions containing 0,5-1x10^5 colony forming units (cfu)/ml.

In a polypropylene microtiter plate a serial dilution series of the protein/peptide to be tested was prepared in 0,01% acetic acid. To 5 µl of every sample, 45 µl of bacterial suspension (0,5-1x10^5 cfu/ml) was added. The plate was incubated shaking (400 rpm) at 37°C. After 2 hours, 0,5 and 10 µl samples were plated on blood agar plates. Bactericidal activity was calculated the next day after colony counting. All experiments were performed in duplicate.

2.2 Bactericidal activity of TC-1* and TC-2.

Bactericidal activity of TC-1* and TC-2 was determined against *E. coli* ML35, *S. aureus* 42D and *B. subtilis* ATCC6633 in killing assays (fig 5).

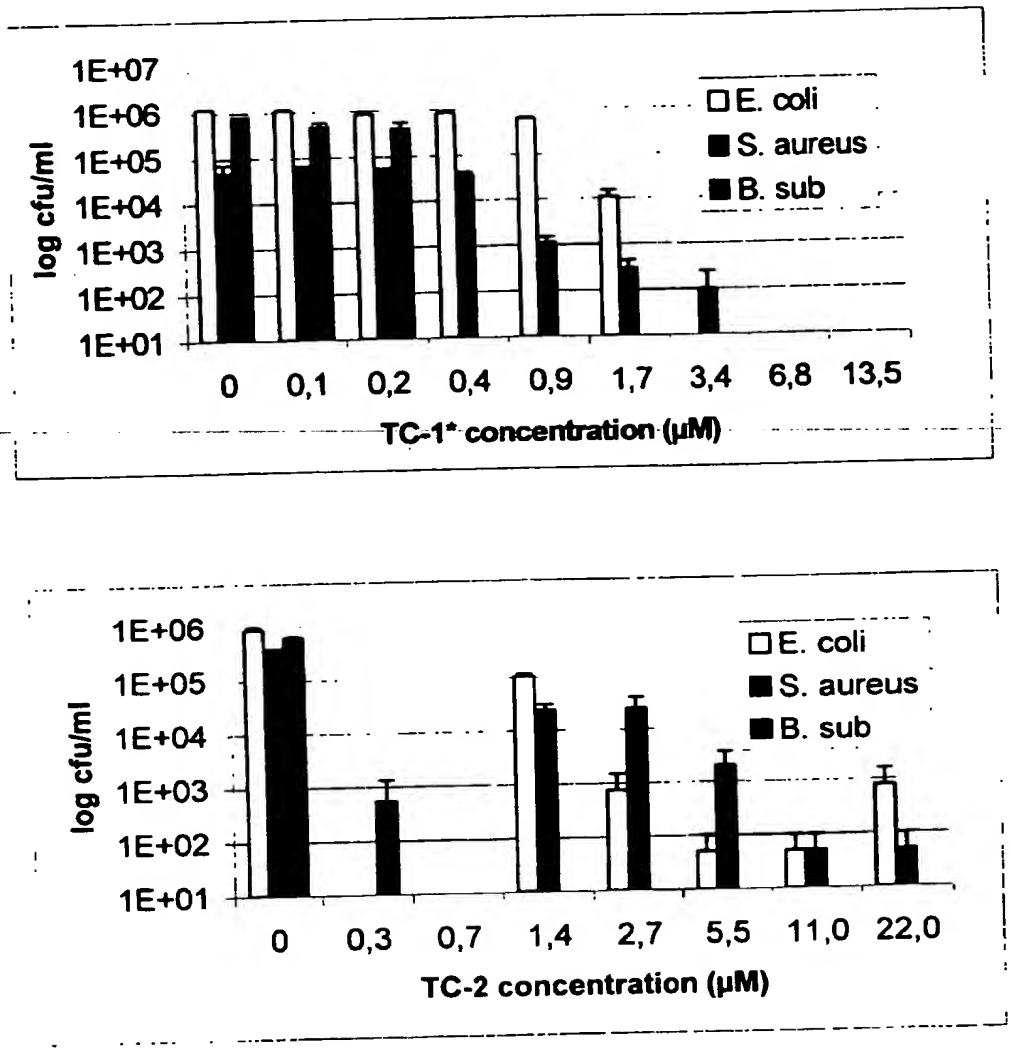

Fig 5. Bactericidal activity of TC-1* (top panel) and TC-2 (bottom panel) against *E. coli* ML35, *S. aureus* 42D and *B. subtilis* ATCC6633. Bacteria (0,5-1*10^5 cfu/ml) were exposed to serially diluted TC (concentrations are indicated). After 2 hours of exposure bacteria were plated and survival was determined by colony counting. Medium: 10mM phosphate buffer pH7,0 + 1% TSB. For TC-2, the 0,3 and 0,7 µM concentrations were not tested against *S. aureus* and *E. coli*.

In fig 5 it can be seen that TC-1* and TC-2 are bactericidal against all three bacteria tested, and that TC-1* is the more active component.

2.3 Bactericidal activity of TC-2 against other bacteria

Bactericidal activity of TC-2 was tested against a panel of other bacterial species. The same method was used as described above, except that 5% BHI (v/v in water) instead of phosphate buffer + TSB was used as a test medium. Bacteria tested were *Streptococcus sanguis* U108 (fig 6), multiresistant *S. aureus* (MRSA), multiresistant *S. epidermidis* (MRSE), *Streptococcus sanguis* J30 (fig 7). This revealed that much higher concentrations of TC-2 were needed to kill U108 than to kill *B. subtilis*. For *Bacillus* (fig 5) 3 µg/ml (0,7 µM) was sufficient to achieve 99,99% killing, where 400 µg/ml TC-2 was required to kill 98% of U108 (fig 6). Exposure of the various bacteria yielded the results given in fig. 7. The MRSA and MRSE are as susceptible to TC-2 as *S. aureus* 42D, while *S. sanguis* J30 seems to be less susceptible.

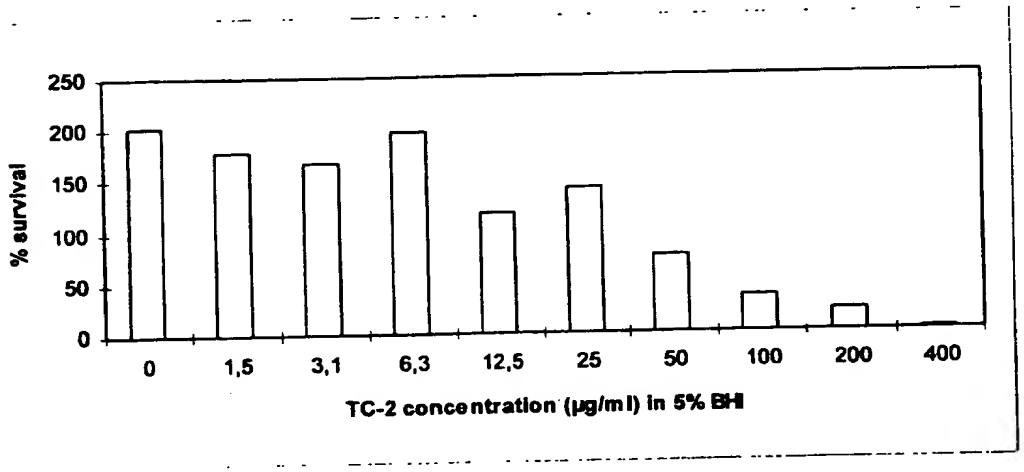

Fig 6. Killing of *S. sanguis* U108 ($1,5x10^4$ cfu/ml) by TC-2 after 2h incubation in 5% BHI.

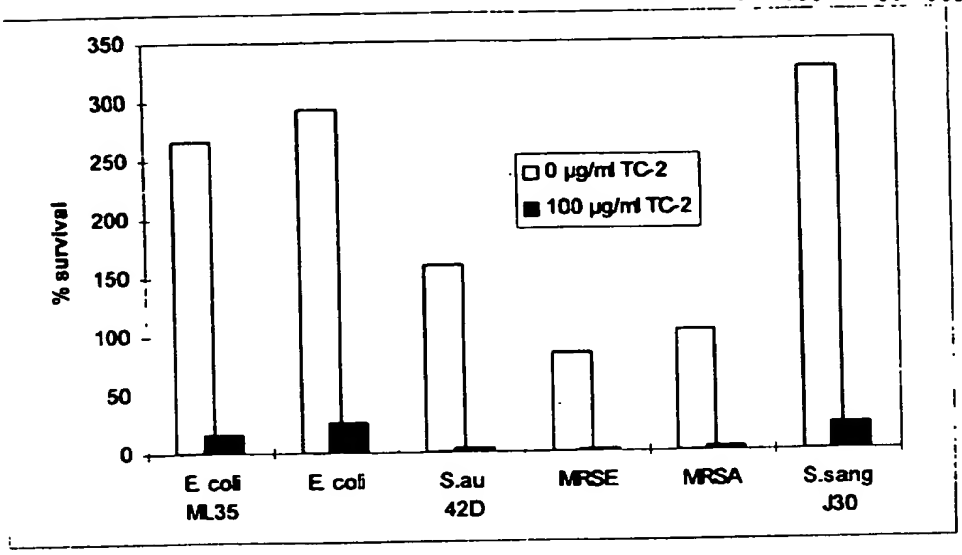

Fig 7: Killing of bacteria ($1\text{-}2x10^5$ cfu/ml) by TC-2 after 2h incubation in 5% BHI.

3. Production and antibacterial activity of recombinant thrombocidins and recombinant CTAP

Several classes of antibacterial proteins contain disulfide bonds. As far as it has been investigated, the presence of these disulfide bonds has been found to be essential for antibacterial activity of HNP-2 (Selsted and Harwig, 1989), GNCPs (Yomogida et al, 1995) and beneficial for activity of protegrins (Harwig et al, 1996). Because disulfide formation is critical for antibacterial activity, a prokaryotic system is not an obvious way to produce these proteins recombinantly. HNP has been produced recombinantly in *E. coli*, but indeed this product had no antibacterial activity, probably due to misfolding of the protein (Piers et al, 1993). Chemokines like NAP-2, CTAP-III (Proudfoot et al, 1997) and IL-8 (Lindley et al, 1988) have been produced in *E. coli*, but these proteins had to be refolded after they had been purified, a procedure which was not needed to observe antibacterial activity of recombinant thrombocidins.

Based on previous analyses of the native proteins TC-1 and TC-2, we designed and produced a number of proteins recombinantly. The proteins produced included rYTC-1, rYNAP and rMCTAP (fig 8a). In brief, this was done as follows. Coding DNA was amplified from a human bone marrow cDNA library by PCR and ligated into Nde1/BamH1 digested pET9a (rMCTAP) or pET16b (rYTC-1 and rYNAP) vectors. Constructs were transformed to E. coli BL21DE3LysS cells, and gene expression was induced with IPTG. Recombinant proteins were purified in 2 steps. rMCTAP was purified by CM-Sepharose cation exchange chromatography followed by preparative AU-PAGE. rYTC-1 and rYNAP were purified using a His-binding column (Novagen) followed by preparative AU-PAGE. The correct structure and purity of rMCTAP-III were confirmed by N-terminal sequencing and MALDI mass spectrometry.

In the last purification step of rYTC-1 and rYNAP, a number of protein bands were identified as the oxidized and reduced forms of these proteins. These different forms were separated and isolated individually.

In addition to this set of proteins two other proteins have been produced: rMTC-1* and rMTC-2. These proteins are identical to native TC-1* and TC-2, respectively, except an additional N-terminal methionine (fig 8b). Like rYTC and rYNAP, rMTC-1* and rMTC-2 were produced in *E. coli* and purified using cationexchange chromatography and preparative AU PAGE. The structure of rMTC-1* andrMTC-2 was confirmed by MALDI and ES mass-spectroscopy. Of rMTC-1*, rMTC-2 and rMCTAP, MBCs were determined for a number of organisms (Table 2). It appears that rMTC-1* and rMTC-2 are bactericidal for *B. subtilis*, although MBCs are higher than for the native proteins (fig 5). The MBC of rMTC-1* for *E. coli* (3,8 μM) is the same as the MBC of the native protein TC-1* (fig 5), whereas the MBC of rMTC-1* for *S. aureus* (15μM) is approximately 2-fold higher than for TC-1* (fig 5). In contrast to this, rMCTAP was not bactericidal for *E. coli*, *B. subtilis* and *S. aureus* at concentrations up to 40 μM. Recombinant NAP-2 (obtained from Bachem, Switzerland) was tested against *B. subtilis* up to 7μM, but no inhibition of survival was observed.

Table 2. MBCs (μM) of recombinant proteins for various bacteria.

	B. subtilis	*E. coli*	*S. aureus*
rMTC-1*	3,8	3,8	15
rMTC-2	7,5		>15
rMCTAP	>40	>30	>30
rNAP-2	>7		

Thrombocidins, Bactericidal Proteins from Human Blood Platelets, are C-Terminal Deletion Products of CXC-Chemokines

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Human blood platelets release bactericidal proteins upon stimulation with thrombin. These proteins may play an important role in host defense in diseases such as endocarditis. Previously we demonstrated that these antibacterial proteins are stored in the $alpha-granules of human platelets, but no further data about their structure and anibacterial activity are available yet. The aim of the present study was to purify and characterize these proteins. We isolated the granules from human platelets by nitrogen cavitation. Antibacterial proteins were purified from the granule content using carboxymethyl-Sepharose cation exchange chromatography and preparative acid urea poly-acrylamide gel electrophoresis. Two proteins were isolated which we designated as thrombocidin (TC)-1 and TC-2. Both of these proteins were characterized by MALDI-tof and electrospray mass spectrometry and partial N-terminal sequencing. These analyses revealed that TC-1 and TC-2 are derivatives of neutrophil-activating peptide (NAP)-2 and connective tissue activating protein (CTAP)-III, respectively, lacking the 2 C-terminal amino acids. NAP-2 and CTAP-III are members of the CXC-chemokine family and are both N-terminal cleavage products of platelet basic protein (PBP). TC-1 and TC-2 were bactericidal against various gram positive and gram negative bacteria. TC-1 was the most active protein with MBCs of 0.4, 3.4 and 6.8 $muM for $\IB. subtilis, E. coli$$END and $\IS. aureus$$END, respectively. Our results present evidence that CXC chemokine-like molecules from human blood platelets are potent bactericidal proteins.

Recombinantly Pr duced Thrombocidins, C-Terminal Truncation Forms CXC-Chemokines CTAP-III and NAP-2, are Potent Bact ricidal Agents.

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Dept. Med. Microbiol, Ac. Med. Ctr, Univ. Amsterdam, Amsterdam, and Dept. Chemical Technology, Univ. Twente, Enschede (1), The Netherlands.

Thrombocidin (TC)-1 and -2 are bactericidal proteins which we isolated from human blood platelets. Structural analyses revealed that TC-1 and TC-2 are derived from NAP-2 and CTAP-III, respectively. Compared to NAP-2, TC-1 lacks the 2 C-terminal amino acids. Likewise, TC-2 lacks these amino acids compared to CTAP-III. In order to obtain large quantities of TC-1 and TC-2 for to evaluate their bactericidal activity in more detail, we produced TC-1 and TC-2 recombinantly. In addition, we produced recombinant CTAP-III to investigate the influence of the C-terminus on bactericidal activity. DNA coding for TC-1, TC-2 and CTAP-III was amplified from a human bone marrow cDNA library and cloned into a $\INdeI$$END/$\IBam$$ENDHI digested pET9a expression vector. The constructs were transformed to $\IE. coli$$ENDBL21DE3LysS cells, and expression of recombinant protein was induced by adding IPTG to growing cultures. Bacteria were harvested, lysed in 6M guanidine, and recombinant proteins were purified to homogeneity in a two-step purification using cationexchange chromatography and preparative acid urea poly-acrylamide gel electrophoresis (AU-PAGE). The structure of the recombinant proteins was confirmed by mass-spectrometry. Routinely, 3-5 mg of purified TC was isolated per liter of growth medium. The MBC of recombinant TC-1 (rTC-1) and native TC-1 (nTC-1) for $\IE. coli$$END was identical (4 µM), whereas the MBC of rTC-1 for $\IS. aureus$$END was 2-fold higher than the MBC of nTC-1 (15 vs. 7,5 $muM). For $\IB. subtilis$$END the MBCs of rTC-1 (3,8 $muM) was approximately 10-fold higher than the MBC of nTC-1 (0,4 $muM). The MBC of rTC-2 for $\IB. subtilis$$END was also higher than the MBC of nTC-2. In contrast to this, recombinant CTAP-III had no bactericidal activity for $\IB. subtilis, E. coli$$END and $\IS. aureus$$END at concentrations up to 40 $muM. Furthermore, the antibacterial effect of NAP-2 for $\IB. subtilis$$END was negligible. In conclusion, we have developed an easy and efficient method to produce thrombocidins recombinantly in $\IE. coli$$END. These rTCs showed bactericidal activity against various bacteria. Furthermore, we have shown that the 2 C-terminal amino acids in CTAP-III and NAP-2 block antibacterial activity in these proteins.

References.

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Selsted, ME, and SSL Harwig (1989): Determination of the disulfide array in the human defensin HNP-2. A covalently cyclized peptide. J. Biol. Chem. 264: 4003-4007.

Yomogida S, I Nagaoka, T Yamashita (1995): Involvement of cysteine residues in the biological activity of the active fragments of guinea pig neutrophil cationic peptides. Infect. Immun 63: 2344-2346.

CLAIM

1. Recombinantly prepared antimicrobial proteins TC-1 and TC-2 or variations thereof, as described in the description.

rYTC-1: MGHHHHHHHHHHSSGHIEGRHM YLRCMCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD

rYNAP: MGHHHHHHHHHHSSGHIEGRHM YAELRCMCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD

rMCTAP: MNLAKGKEESLDSDLYAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DESAD

Fig 8a. Recombinantly produced proteins.

rMTC-1*: MAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DES

rMTC-2: MNLAKGKEESLDSDLYAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAG DES

Fig 8b. Recombinant proteins currently being produced.

Antibacterial activity of rYTC-1, rYNAP-2 and rMCTAP-III was determined in a radial diffusion assay with *B. subtilis* as the test organism (fig 9). Oxidized and reduced forms of rYTC-1 and rYNAP are included.

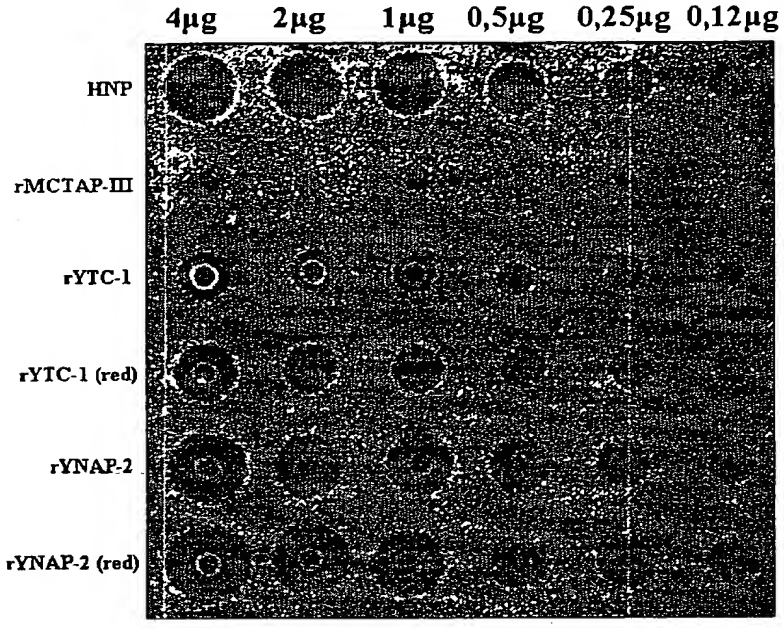

Fig 9. Antibacterial activity of rMCTAP-III, rYTC-1 and rYNAP-2 in radial diffusion assay. HNP was used as a positive control. (red): reduced form of the protein indicated. Amounts of protein added to each well are indicated. Test organism: *Bacillus subtilis*.